9 Assessing Effects Through Semi-Field and Field Toxicity Testing

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9.1 INTRODUCTION

Semi-field and field studies may be conducted for regulatory purposes if lower tier assessments trigger further evaluation of a chemical's potential to cause adverse effects. For example, a regulatory trigger value may have been breached in the lower tier assessment that in turn means that a protection goal may not be met based on the findings at that level. One way to ensure that a protection goal is met is to modify the use of the subject compound such that it may no longer pose an unacceptable risk to the honey bees *Apis mellifera*[1] and/or non-*Apis* bees.[2] However, modifying or restricting the use of a compound may be undesirable or unnecessary if further information is obtained from either a semi-field or field study that demonstrate otherwise. Such a study or studies should provide greater insight into whether adverse effects to *Apis* and/or non-*Apis* bees are likely to occur under real-world field use of the pesticide in question. As such, the objective of the regulatory study(ies) may be to try to indicate, both quantitatively and qualitatively, what the possible effects may be under more environmentally realistic or relevant conditions. Such studies should be predicated on a well-developed problem formulation that builds on lower tier studies as well as the associated risk assessment.

As part of problem formulation, there should be a clear idea of the regulatory concern, identification of protection goals, assessment endpoints and related measurement endpoints on which to base judgments. For the purpose of developing guidance relative to higher tier tests, the participants of the Workshop assumed the protection goals stated at the outset of the conference, which include:

1. Protection of managed pollination in agricultural/horticultural-based crops (i.e., *Apis* and non-*Apis* species)
2. Protection of honey production and other hive-products; and
3. Protection of biodiversity (primarily non-*Apis* bees).

This chapter provides an overview of what to consider when planning or assessing either a semi-field or field study. As regards the honey bee, much use has been made of European Mediterranean Plan Protection Organization (EPPO) 170 (EPPO, 2010) and Organization for Economic Cooperation and Development (OECD) 75 (OECD, 2007). Participants during the Society of Environmental Toxicology and Chemistry (SETAC) 2011 Workshop used their collective practical and regulatory experience to provide further information on how a study should be conducted. Therefore, the following is seen as a development of both EPPO 170 and OECD 75 based on the experience of the experts present at the Workshop. If the risk assessor indicates the need for either a semi-field or field study, then it is recommended that this chapter along with the information provided in EPPO 170 and OECD 75 be consulted. The information in these references may also be consulted when such studies are being evaluated for regulatory purposes.

[1] It should be noted that when referring to *Apis mellifera*, we are referring to approximately 17 subspecies that originated in Europe.
[2] Non-*Apis* bees are highly varied in terms of social and solitary lives, the duration of their activity in the field, the amount of pollen and nectar they store, and where they nest. For details, see Chapter 3 and Chapter 8.

9.2 DEFINITION OF SEMI-FIELD AND FIELD STUDIES

Elements in the design of semi-field and field studies encompass the study's objectives, the test organism, a study site, methods, endpoints, sample design, quality assurance/quality control standards, and the statistical analysis of the data. In discussing the elements of a semi-field study, the participants of the Workshop defined a semi-field study as the following.

A **semi-field study** is designed to measure exposure and/or effects and is performed on a crop that is grown outdoors in an enclosed test system with controlled or confined exposure. The crop is subject to good agricultural practices (i.e., grower standard practices), and therefore, there will or could be weeds present but the predominant plant, and thus the source of nectar/pollen, will be the crop. The test system could nevertheless be designed to reflect a desired exposure system and specific foraging environments, for example, a mixture of crop and weeds, or flowering margins. The details of the test design (such as application parameters or measurement endpoints) will depend upon the regulatory questions being asked. However, semi-field studies generally attempt to maximize exposure by confining bees to a particular source of treated nectar/pollen.

For species (both non-*Apis* and *Apis* ~~species~~) that are used to pollinate plants grown in greenhouses, it may also be necessary to carry out a higher tier study. A semi-field study will be enclosed with controlled or confined exposure but will be of reduced size compared to a commercial glasshouse. The size of the test environment is related to the species being studied, and the questions or issues being investigated.

A semi-field study, therefore, provides for a potentially worst-case exposure scenario.

A **field study** is designed to measure exposure and/or effects and is performed on a crop that is grown outdoors with no enclosure. The crop is established and maintained following good agricultural practices. While the bees are free-flying and able to seek out alternative food sources, alternative sources of pollen and nectar should be minimized. The study design elements (e.g., selection of crop, duration of the study, or environmental conditions) will depend upon the questions being asked. A field study for a greenhouse situation should be conducted in a commercial greenhouse.

9.3 DESIGN OF A SEMI-FIELD STUDY

When deciding whether a semi-field study is appropriate, it is necessary to consider various strengths and weaknesses of this type of study to ascertain whether it is the most appropriate way to refine the understanding of the potential risks from the use of a compound. Outlined below in Tables 9.1 and 9.2 are the strengths and weaknesses of semi-field studies for *Apis* and non-*Apis* bees.

9.3.1 When would a Semi-field study be appropriate?

Consistent with the tiered approach to toxicity testing and risk assessment, semi-field studies may be triggered when lower tier assessments (relying on laboratory results) indicate potential risks that are inconsistent with protection goals. In such cases, higher tier tests may provide information that reduces the uncertainty about risk, allowing for a more informed decision. Outlined below are scenarios when a semi-field study may be appropriate; and when a semi-field study may not be an appropriate option.

- If, as a result of the initial laboratory assessment, acute mortality and/or sub-lethal effects are considered to be the main concern, then a semi-field study may be appropriate.
- If repellency or an impact on foraging activity is predicted, either on the basis of efficacy data (e.g., a compound is known to act via an antifeedant effect) or from any observations, laboratory or any other relevant studies, then a semi-field study may give the risk assessor useful information on the

TABLE 9.1
Strengths and Weaknesses of Semi-Field Tests with *Apis Mellifera*

Strengths

Exposure is known since the bees are enclosed and there is usually a toxic reference treatment group. (The toxic reference treatment (using chemical of known toxicity to bees) is used to confirm that the bees are exposed to the treatment and to calibrate the ability of study to detect treatment effects known to be associated with the reference chemical.)

Provides realistic exposure both inside and outside the hive, that is, to both materials available at the target crop as well as concentrations in the hive.

The test system can also be designed to determine the residual toxicity. Weathering of the applied material and natural exposure of honey bees are inherent in the design.

Irrigation of the crop (via drip irrigation to avoid wash-off) is possible, hence potentially reducing the likelihood of the study being adversely affected by drought.

In contrast to laboratory studies, semi-field studies present a more realistic scenario of interaction between the bees and the environment.

Due to their smaller size and shorter duration, semi-field studies are less affected by fluctuations in ecological variables.

Potential for ~~sub-lethal~~ effects can be observed more easily than in either laboratory or field studies.

Brood can be considered in specifically designed semi-field studies (see OECD 75).

Semi-field tests are relatively quick and easy to perform.

Semi-field environments are smaller scale in operation than field studies, making it feasible to test greater numbers of replicates, which in turn should allow for more robust statistical designs.

As the bees are enclosed and have no alternative foraging environment, the exposure is potentially a "worst-case" scenario.

Certain exposure scenarios that are difficult to study under real field conditions, for example, aphid honeydew, can be studied under semi-field conditions.

Weaknesses

Experience with performing these studies has shown that it is difficult to keep colonies in an enclosed structure for long periods and, as a result there is a limited amount of time that a colony of *A. mellifera* can survive in the enclosure. The correct stage of crop bloom is critical to the study and, as a result it is only appropriate to assess the effect of short-term exposure including potential effects on brood (~~see~~ OECD ~~75~~). Where exposure is either repeated over a sustained period (e.g., where there are repeated applications of the pesticide), or where exposure is continuous (e.g., from the use of systemic seed treatment), semi-field studies may be of limited usefulness in determining long-term effects.

Semi-field studies tend to use colonies with only 3000–5000 bees (EPPO ~~170~~), which is smaller than a full size (managed) colony. Due to the current state of knowledge, it is not possible to determine whether an observed effect in a semi-field study will result in either an effect in a standard full size colony or an effect under field conditions. Hence, extrapolation of adverse effects to a full size unenclosed colony under more realistic field conditions may not be possible.

Due to the small size of the colony, it is not as easy to assess pollen and nectar storage and hive weight development; therefore, it is difficult to assess potential effects on honey production (i.e., a potential protection goal identified at the Workshop) when adverse effects are observed on other parameters.

Because the size of the colonies used in semi-field studies prohibit their ability to successfully overwinter, these studies may not provide information on overwintering success.

Due to the nature of the enclosed test design, not all crop scenarios are possible to test, (e.g., size of plants, area required, and nutritional value of crop to bees).

There is potentially limited foraging area; therefore, care is needed to ensure that sufficient nutrition (i.e., enclosed crop area) is available.

There is a possible stress on bees due to enclosed nature of the study, that is, bees have a desire to escape, consequently reducing their foraging activity on the crop. However, balance of tent size/crop field size and colony size should ensure foraging and exposure (~~see~~ EPPO ~~170~~).

TABLE 9.2
Strengths and Weaknesses of Semi-Field Tests with Non-*Apis* Bee Species

Strengths

Individual colonies, or aggregations of individual solitary bees (such as *Meloponini* or *Bombus*) can be used and thus the pesticide effects are readily interpreted. Increased replications are possible and readily performed so statistical analysis may be easier.

Product use on a wide range of crops, including those that are not readily pollinated by honey bees (e.g., eggplant), can be assessed.

Some social non-*Apis* bees amenable to these tests, such as *Meloponini* (stingless bees) and *Bombus*, are easier to handle than *Apis* as they are reluctant to sting. Additionally, many of the solitary non-*Apis* bees, although capable, are reluctant to sting. Solitary bee species amenable to semi-field studies (e.g., *Osmia* and *Megachile* species) will not sting.

The area of the enclosure of a semi-field study can support full colonies of non-*Apis* species (*Bombus* or *Meloponini*) or a collection of independent individuals (solitary bees), hence an extended study can be done. These bees have a complete life cycle in 3–6 weeks (solitary bees) or one season (*Bombus*) in temperate climate.

Individual solitary bees typically provision nests over a 3–6-week period, thus allowing for a complete (or at least almost complete) life-cycle study for solitary bees if the forage crop flowers for more than 3 weeks.

It is possible to do larval exposure tests with solitary bees because pollen/nectar is brought straight to a cell and an egg is laid on the nest. This behavior leads to a potentially conservative assessment since the progeny has direct exposure, dermal and oral, with food resources that potentially contain the test pesticide.

Non-*Apis* bees can be used and maintained efficiently in small enclosures.

Non-*Apis* bees will forage under less optimal conditions in terms of temperature, relative humidity, and wind. This is especially true for *Osmia* and *Bombus* spp., which are quite hardy.

In solitary species such as those in Megachilidae, the larvae are in direct contact with nectar and pollen, and so there is the possibility of contact and oral exposure. This is not the case with *Apis* larvae that require a special larval test to ensure adequate expose larvae to a given pesticide and route of exposure.

Weaknesses

Resource supplements may be needed for crops that do not provide both pollen and nectar, which may reduce bee activity.

In temperate areas, the annual life cycle of solitary bees limits the window in which adult or larval testing may be conducted.

There is significant uncertainty as to how representative the current commercially available non-*Apis* bees are for other non-*Apis* species. For all non-*Apis* bees, there is enormous variation in the use of resources, behaviour, habitat requirements, life cycles, etc.

potential short-term effects the compound may exert on foraging behavior. Due to the confined nature of the study, ~~it can be concluded that no~~ effects in a semi-field study ~~is~~ indicative of ~~no~~ short-term effects ~~under~~ situations. However, if a potentially significant adverse effect on foraging behavior is observed, then there could be long-term effects and it may be necessary to extend the semi-field study or conduct a full field study.

- A semi-field test may be used to validate or test a safe re-entry time for bees. Based on information gathered from a foliar residue toxicity study (see Chapter 8), a semi-field test can be used to provide additional information on the residual toxicity of a compound under more environmentally relevant conditions. For example, a semi-field study may provide information on the test compound residues, (i.e., when residues are dry and therefore "safe" for bees). This information can be applied to risk mitigation.
- If a pesticide is systemic and intended to be used as either a seed treatment, solid formulation (e.g., granule or pellet), or soil treatment, then a semi-field study can provide detailed information regarding exposure levels both in the target crop and in the hive associated with the specific application

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parameters. Care is required in selecting a study site to ensure that environmental conditions (e.g., soil conditions (moisture, pH), duration from soil treatment to drilling, and flowering) are appropriately representative of the proposed use. The study can also provide an indication of the likelihood of initial mortality and initial behavioral effects following exposure. Since confinement may affect bee behavior per se, it is necessary to compare effects seen in treatment groups with those observed in the control. If there is a possibility of long-term effects resulting from this type of exposure, then it may be possible to modify this study appropriately or alternatively it may be preferable to conduct a field study. It is also important to target the exposure position of the study (i.e., the portion when the colonies are confined under an enclosure) with the time when the test plant is flowering and the highest expected residues are present in pollen and nectar.

- If the compound is an insect growth regulator, or exhibits insect growth regulatory characteristics, then a test according to Oomen et al. (1992) or a semi-field study over a 28-day period (OECD 75) can provide information on the potential effects on growth or development.

One of the advantages of a semi-field study, in comparison to a field study, is that it allows for the inclusion of a toxic standard (i.e., one replicate is run with a test material that is known to elicit adverse effects to the test organism). However, since there are occasions where it is not possible to use a toxic reference chemical (e.g., systemic seed treatments[3]), the absence of a toxic reference does not greatly compromise the utility of the test. When testing seed treatment application scenarios, the residues on treated seed should be determined as well as the residues in pollen and nectar; exposure to the bees is assumed as the test system is closed and exposure cannot be avoided.

- Semi-field studies are also useful studies for non-*Apis* species such as *Megachile rotundata* as they may provide information on alternative routes of exposure, that is, leaves which are used for nest building, in addition to conventional routes of exposure such as nectar and pollen.
- It is possible to determine colony effects in a semi-field study over an extended period (e.g., for 3 months or longer) with species such as stingless bee and bumblebee colonies. For example, a bumblebee colony may be housed in a box with two connected chambers (one chamber for the colony's nest and one chamber from which the colony may be fed (Kearns and Thompson, 2001). The nest box may be opened and the colony allowed to forage outside in a semi-field enclosure. After this exposure period, the nest may be closed and the colony fed in the nest box's feeding chamber for a month or two to look at delayed lethal or sub-lethal effects on reproduction and colony growth. After a couple of months, bumblebee colonies will switch from raising workers to raising drones and queens. Similarly, one can expose foragers from a stingless bee colony for several days in a semi-field enclosure and then close up the nest box. The colonies in this case can then be fed by placing food (sugar water and vitamins) at regular intervals into the nest box. Stingless bees have perennial colonies (much like honey bees) and may be fed *in situ* for many months.

As noted above, semi-field studies address mortality from short-term exposure as well as short-term behavioral effects. However, there is a concern whether they are able to address, (i) long-term effects from either short-term/sub-lethal exposure or, (ii) long-term effects from long-term/continual (i.e., via hive products) exposure or long-term chronic exposure.

[3] The lack of a toxic standard for a systemic seed treatment or solid formulation is due to the lack of a compound that causes known effects.

9.4 OUTLINE OF A SEMI-FIELD STUDY FOR *APIS* AND NON-*APIS* BEES

9.4.1 Design of a semi-field study for *Apis* bees

The following section is based largely on EPPO 170 ~~(2010)~~ and OECD 75 and should be seen as an extension of both the guidance documents, and considered along with the details of these guidances. In developing the elements of this chapter, the Workshop participants relied upon their experience as well as information included in EPPO 170 and OECD 75. The aim of the following section is to highlight further issues to be considered when planning and carrying out a semi-field study as well as issues that should be considered when evaluating a semi-field study for risk assessment purposes.

It is important that the aims of any semi-field study are clearly determined prior to the conduct of these studies. Clear problem formulation is required to ensure that the study is appropriately designed and focused to address the regulatory questions being asked. All semi-field studies should be designed to address specific concerns highlighted at lower tiers. EPPO 170 and OECD 75 are relatively flexible guidance documents and consequently allow studies to be designed to address specific issues. The considerations of the participants of the workshop, and of this chapter, do not remove or reduce that flexibility of the referenced ~~EPPO or OECD~~ documents, rather they highlight areas or elements that are thought to be important considerations for incorporation into a semi-field study.

9.4.1.1 Size of Semi-Field Study

The minimum size of a semi-field study enclosure according to EPPO 170 is 40 m^2. Recommendations in this section are based on professional experience and are considered appropriate in terms of practicality of conducting the study and for determining effects of mortality and behavior. However, this area is only appropriate in terms of certain field crops (e.g., *Phacelia*, oilseed rape/canola, mustard). For other crops (e.g., melons, apples), the area (40 m^2) may need to be amended due to issues such as the number, density, and attractiveness of flowers, availability of nectar and pollen, or the size of the plants. The area of the test enclosure may also need to be amended depending upon the size of the colonies being used.

It should be noted that when studying bee brood, an increased (enclosed) crop area (>60 m^2) may be preferable to ensure the colony has access to adequate floral resources. However, the precise area depends on colony size, crop, and duration of confinement; 40 m^2 (OECD ~~75~~) may be acceptable for a small colony that is confined for no more than 10 days.

9.4.1.2 Crop

The standard crops (i.e., oilseed rape~~/~~canola, mustard, and *Phacelia*) are easy to cultivate and manage but more importantly are highly attractive to honey bees. *Phacelia* has an open flower that it is highly attractive. The openness of its flower will mean that bee-relevant parts of the flower will be fully exposed to the spray application; hence, honey bees foraging after the spray application will be exposed to residues. Oilseed rape and mustard are both highly attractive to honey bees so a high level of exposure can be ensured. Results from studies carried out on these crops can be extrapolated to other crops, provided that the application parameters in terms of application rate, timing of applications, and number of applications used on the surrogate crops are comparable (ideally identical) to that of the subject product. If effects are observed on these standard crops then it may be possible to further refine the assessment by using the target crop species.

When considering systemic soil or seed treatments, it is preferable to use the actual~~/relevant~~ crop. A crop other than the target crop needs to be justified on the basis of exposure (e.g., it may be appropriate to select a crop that is attractive and has high residues in nectar and pollen as a "model" crop rather than the actual crop of concern).

9.4.1.3 Size of Colony

Each tunnel/cage/tent should include one, healthy queenright (i.e., a fertile, laying queen) colony per cage. Precise size of the colony used will depend upon the study design; EPPO recommends a size of 3000–5000 bees.

It is important to have sufficient nutritional resources within an enclosure to ensure that the bees do not starve. Generally, feeding will not be necessary; however, if there is concern regarding the attractiveness of a specific crop/situation, then supplemental feeding may be needed. For example, if testing maize, then additional food will be required as maize produces no nectar.

9.4.1.4 Test Treatment

Sprays Only Test treatments and water (negative) controls are required; ideally a positive control (reference toxicant) is also required. It is customary to test only the proposed field rate. If, however, a model crop is used, for example, *Phacelia*, then it may be appropriate to have more than one treatment rate. This may enable the data to be extrapolated to other crops and other application rates. Additional tunnels/cages could be used to address different application rates as well as effects from treating at different times of the day. However, at a minimum, a study at the maximum proposed rate should be carried out.

A positive (reference toxicant) control: (i) provides an indication of the sensitivity of the test system; (ii) demonstrates exposure; and (iii) indicates the magnitude of response to a known toxin. However, positive controls kill bees unnecessarily and can add to the cost and complexity of study design; therefore, their use should be considered carefully. Positive control compounds are useful if it is unclear if any dose of the tested pesticide will have effects. If a positive control is used, it is necessary to select a compound whose toxicity profile is known and consistent with that under consideration, for example, for assessment of a potential acutely toxic compound, then there is a need to use a similar compound. Historically, dimethoate has been used as a reference chemical when studying acutely toxic compounds on adult forage bees. If insect growth regulatory effects are expected, then a known insect growth regulator with similar effects should be used. When a positive control is used, there should always be clear effects. There should not be sustained mortality at high levels in the water control. There should be an appropriate number of replicates for the treatment groups to provide sufficient power to discriminate treatment effects with a level of precision.

For Systemic Solid Formulation/Seed Treatments/Soil Treatments While there is a need to have both the treatment and a water (negative) control, currently it is not possible at this time to identify a suitable positive (reference toxicant) control for most systemic solid formulation/seed treatments/soil treatments.

9.4.1.5 Pre-Application

Sprays Only Healthy colonies should be used and transferred to the test site a minimum of 2–3 days prior to treatment. This is due to mortality that inevitably occurs when a colony is moved and subsequently confined. If the hive is moved during the day, the hive will tend to acclimate more quickly. There should be a measurement of mortality over the acclimation period; the greater number of measurements of mortality will provide greater confidence that the effects after treatment are attributable to the treatment rather than due to the hive acclimation. It is likely that there will be variability between colonies and every effort should be made to ensure that they are as consistent as possible. This can be partly achieved by moving the colonies at the same time. Attempts should be made to ~~make sure that~~ the colonies are as similar as possible, in terms of the number of bees, at the start of the study. Excessive variation at the start of the study will make the study difficult to interpret and hence potentially limit~~s~~ its usefulness.

Further work is required to determine the range of background levels of mortality once the colony(ies) is situated at the test location in order to establish acceptable levels or ranges of mortality. These background levels could be used to help interpret whether the level of mortality observed in the treatment is treatment-related or not, providing an indication as to the overall reliability of the study.

With spray treatments, the colony is placed in the semi-field setting when the crop is just about, or at flowering. The effects of the pesticide to honey bees foraging that crop are then determined. With systemic chemistries, there is a potential for exposure to occur over a longer period of time; therefore, the honey bees should be present during the whole flowering period of the plant. Acclimation as outlined above is, therefore, not possible as exposure of the bees to the pesticide will occur as soon as they are introduced into the treatment area. However, a consideration of mortality due to moving the colony is still required. One potential way around this problem is to compare the mortality that occurs with the untreated crop to that with the treated crop. Nevertheless, the significance should be determined statistically. Semi-field studies may be most effective for determining acute effects related to systemic chemistries. If sub-lethal effects are predicted, then a modified semi-field designed to ascertain any long-term effects, or simply a full-field test may be more appropriate.

9.4.1.6 Post-Treatment Assessments

Assessments of mortality via the placement of dead bee traps, sheets, or tarps at the front of the hive and within the enclosure should ideally be carried out daily but at least on days 0, 1, 2, 4 and 7 post treatment. This frequency is not appropriate for in-hive assessments as the disturbance could cause significant effects.

Sub-Lethal Behavioral Tests There is a need to standardize and refine the number and type of tests or observations that can be made to document potential behavioral changes due to sub-lethal pesticide exposure. In these tests, it is typical to report abnormal behavior in foraging or other behaviors that may be exhibited during the test; but, definitive and meaningful quantifiable measures may often be lacking. Rather than making general observations on bee behavior, it is proposed that more detailed measurements can be made in addition to the general observations used to date. Of these, perhaps the most obvious is in measuring foraging activity.

When measuring foraging activity, the number of returning foragers should be counted before treatment and at regular intervals post treatment. The number of returning foragers with pollen loads should constitute a separate count from those returning without pollen (nectar and water foragers). Observations should last for 1–3 minutes. The observation periods should be equally divided across all test groups so that measurements are taken at approximately the same time with the controls as with treatments.

A second observation that could be quantitatively measured in a semi-field test is the average flower handling time. This measure is made by recording the time taken for the bee to work a flower (i.e., to remove pollen and/or nectar). The observer simply records the total flower handling time for bees collecting pollen and nectar. If the flower type is such that distinct pollen and nectar foraging is possible, then these forager types should be kept separate. The exact number of required measurements should be determined or justified statistically. The time of day at which measurements are taken should be randomized between plots to avoid time of day and or weather bias. As with previous studies of this type, general observations of any unusual bee behavior should be noted and quantified if possible (e.g., 30 bees were seen twitching and exhibiting excessive grooming on the landing board during the 1–3-minute foraging counts). In addition, it may be possible to determine foraging behavior in front of the hive.

Due to the confined nature of semi-field studies, it was the consensus of the Workshop participants that an adverse effect on behavior compared to the control should be interpreted with caution and should trigger additional consideration. The relevance of an effect, or lack thereof, in a semi-field study may not be assumed to be directly translated to the field scale. Interpretation of effects, or lack thereof, must be done with care. Additional information could be obtained to aid interpretation of any effects seen. This information could come from a variety of sources; however, the Workshop participants considered that field studies were the most appropriate source to validate any effects or lack of effects that are considered significant.

Depending upon the regulatory question being asked, it may be necessary to determine residues in fresh pollen, stored pollen, nectar, honey, and wax. The types of samples to be collected depend on the study and the questions to be answered. Residues in foraging honey bees may also be ascertained and this information could be used in interpreting potential incidents.

9.4.1.7 Results

Traditionally when determining if a study is acceptable, there is consideration of whether it has met various quality criteria, such as adequate controls or chain of custody. In addition, there should be consideration as to how the study compares to the above guidance. The use of a toxic reference chemical can help meet the need for quality assurance measures; however, it is not essential for the reasons stated above.

Based upon the study objectives, key outputs from a standard semi-field study could be the following.

- Mortality in the crop: use of sheets or tarps in the crop.
- Mortality at the hive: use of dead bee traps or sheets in front of the hives.
- Foraging activity and other behavior.
- Measures of exposure: residues in pollen, nectar, pollen pellets, and dead bees.
- Pollination deficit: it may be possible to determine if there is a difference in the degree of pollination success (e.g., via fruit set) of the treated versus untreated crop.
- Assessment of the brood, including an estimate of adults, the area containing cells, larvae, and capped cells (if this is a key area then methods outlined in OECD 75 should be followed).

9.4.2 Design of a Semi-field Study for Non-*Apis* bees

At present, there is no equivalent EPPO 170 or OECD 75 guideline for using non-*Apis* bees in semi-field or field studies. As a result, the Workshop participants suggest that if there is a regulatory question regarding a pesticide that requires the inclusion of a non-*Apis* species as a result of triggers activated by laboratory effects bioassays, the study design should be developed on a case-by-case basis with consideration of the specific endpoints described for semi-field honey bee studies and the overall regulatory question. Care should be taken when evaluating and interpreting results from these studies until protocols are sufficiently vetted through ring-testing.

When selecting non-*Apis* species to be used for semi-field studies, attention needs to be paid to their availability, ease of handling, and survival under experimental conditions. Therefore, it is recommended that the species used are those that are either commercially available or can be readily reared under laboratory conditions.

9.4.2.1 Semi-Field Studies—Solitary Bees

Three solitary non-social bee species are recommended for use in semi-field studies in temperate zones: *Osmia lignaria*, *Osmia Bicornis*, and *M. rotundata* (Johansen et al., 1984; Tasei et al., 1988; Konrad et al., 2008; Ladurner et al., 2008). *M. rotundata* will be used as the descriptive species in this section.

M. rotundata, the alfalfa leafcutting bee, is a non-social Eurasian bee species that is widely managed as a pollinator of alfalfa for seed production in the United States and Canada, and is occasionally deployed for the pollination of other specialty crops (e.g., canola, carrot—for seed, blueberries). Dormant alfalfa leafcutting prepupae are sold as loose cells in 4 L increments (approximately 10 000 individual cells).

Due to standard field production cycles, dormant loose cells are usually only available from late fall through early winter. Cells should be maintained at 1.7°C–4.4°C and 50% relative humidity (RH) until natural emergence during early summer in most of the northern hemisphere. Bees maintained in cold storage beyond this point begin to deplete stored energy reserves and may fail to emerge upon incubation (210 total days is the general upper limit for diapause before viability declines significantly). Cells should be stored in open or ventilated containers and tumbled periodically to reduce the growth of molds. Bees can be incubated to adulthood with as few as 150 days of cold storage diapause. Careful control of temperature (i.e., 29°C) and humidity (70% RH) will cause most of the incubated bees to emerge from their cocoons at approximately the same time (50% emergence in 23 days and complete emergence after 32 days).

Few release rates (density rates) exist for crops with the exception of alfalfa, where 74 000 to 100 000 bees per hectare are recommended, and canola and blueberries, where 50 000 bees per hectare (Mader et al., 2010a) are recommended. Release rates will vary based on the size of enclosure and crop to be utilized in the semi-field study but could be as few as 200–500 solitary bees per tunnel site of 40 m^2.

Site selection for the study should use the same criteria as those for semi-field *Apis* studies. Once an enclosure is ready, a wooden nest shelter containing enough styrofoam nesting boards to accommodate all the *M. rotundata* to be released for the study should be placed in the test enclosure (2–3 nest tunnels per bee), facing the morning sun, 3–4 days in advance of the initiation of the study (i.e., before the pesticide is to be sprayed in the semi-field enclosure). Bees ready to emerge or already emerged should be placed in front of the nest shelter and left to orient to the nest. Bees should not require supplemental feed as long as there is sufficient crop in bloom. These bees do not require a water source so long as enough flowers or a nectar feeder is available. However, if mason bees (*O. lignaria*) are used, a drip bucket and excavated damp mud pit are needed inside a test enclosure (i.e., tunnel) cage. The mud pit should be excavated so the bees can access the soil profile layer with the best clay-water content. Nectar is not sufficient for wetting mud.

Key Outputs

- Mortality in the crop: same as for *Apis*.
- Mortality in the hive/nest shelter: use of a tarp placed on the ground in front of the nest shelter may allow some assessment of *M. rotundata* mortality. However, solitary bees may die within the nest material, making mortality assessment more difficult. Assessment schedule should be the same as those for ~~*A. mellifera*~~
- Foraging activity: same as for *Apis*.
- Reproductive success (colony health): once it is known that the released female *M. rotundata* have successfully mated and started to provision cells (i.e., individual cells/eggs are present or tunnels are sealed), assessments on increasing brood nest (e.g., brood development) can begin. Nest boxes can be monitored on the first day once cell provisioning has commenced and continued on a weekly or bi-weekly basis. Count and mark completed tunnels. Observation nests (grooved boards with clear acetate or glass covering the grooves) can be used to observe nest, cell, and brood development without disturbing the bees. At 15.6°C (60°F), eggs of *M. rotundata* take 15 days to hatch and then an additional 35 days are required for the larvae to reach the prepupal stage. At 35°C (95°F), it takes 2–3 days for the eggs to hatch and 11 days for the larvae to reach the prepupal stage (Mader et al., 2010a). Therefore, if flowering of the study crop ends prior to either 14 days at 35°C or 50 days at 15.6°C, then the nest box needs to be removed from the study site and placed in a growth chamber that simulates the average temperatures experienced by the bees while they were in the enclosure. Once the prepupal stage has been reached, a segment of the styrofoam nest needs to be dismantled, and cells per tunnel counted and weighed, and then dissected to determine the number of cells with prepupae and those that are provisioned but with no larvae present. If there are no larvae present (i.e., these cells are called "pollen balls"), it indicates that larvae have died in the first or second larval instar, which may be related to the exposure to extreme temperatures (cold and hot) during that stage in development (Mader et al., 2010a). The remaining styrofoam nest sections can be dismantled, cells counted and then placed in storage at 2°C–5°C (35°F–40°F) at 50% RH until the following

spring. At that time, the diapause can be broken and the number of emerged adults can be counted and compared to the total number of cells. This allows for determination of mortality in progeny (sub-lethal effects).

9.4.2.2 Semi-Field Studies—Social Non-*Apis* Bees

Bombus spp. will be used as the descriptive species in this section.

Bumblebee colonies are readily available from commercial sources.[4] A colony consisting of 50–300 workers and a queen can efficiently pollinate 1000–3000 m^2 (Morandin et al., 2001) of tomatoes, yet should also perform as well as a honey bee nucleus hive in a smaller enclosure (40–60 m^2). The 40–60 m^2 foraging area, and considerations for supplying alternative forage (e.g., nectar or pollen) are relevant considerations for bumblebees, as they are for honey bees. In addition, feeding bumblebee colonies can be done in a much more controlled way than *Apis*. When *Bombus* are commercially reared, they are fed in the nest, and the same could be done for colonies used in a semi-field test. Colonies should be provided with identical amounts of supplemental pollen and/or nectar, helping to minimize differences between treatments. Also, when changing food stores, the pollen or nectar that was not consumed can be removed and weighed in order to determine how much the colony consumed. A colony population of at least 100 workers and a queen should be used for semi-field studies, and exposure duration should be 10 days followed by supplemental feeding. If the colony is movable, then it may be appropriate to move it to a non-agricultural and pesticide-free landscape to continue development outside the tunnel, rather than keep them inside the tunnel with artificial food.

When extracting bees for sampling, or for mark and release, it is necessary to distinguish the queen (usually the largest bee) from the workers. Harm to the queen is likely to result in defensive behavior on the part of the workers and a rapid reduction in colony life span. Similarly, it may be desirable to distinguish between male bees and female workers. In general, male bumblebees have larger eyes, longer antennae, no pollen baskets (corbiculae), and depending on the species, may have a notable patch of yellow hair on the front of their face.

One to two *Bombus* spp. colonies of similar age and with approximately 300 workers per colony should be moved to the semi-field study enclosure with entrances closed in the morning. Each colony should be placed on a concrete block with the entrance facing the morning sun. This should be done 2–3 days prior to the initiation of the study.

Key Outputs

- Mortality in the crop: same as for *Apis*.
- Mortality at the hive: same as for *Apis*. A small tarp can be placed under the colony extending outward from the entrance so that any dead adults or drone larvae discarded by the colony can be counted over time. The tarp should be cleaned of all discarded adults and drone larvae after each assessment. Endpoints such as discarded dead adults and drone larvae are indicators of colony condition.
- Foraging activity: same as for *Apis*.
- Reproductive success (colony health). Before placing colonies in the semi-field enclosure, a (close-up) photograph should be taken of the brood nest and food stores through the plastic inner cover at night when most of the bees are back in the nest. The photograph should be labeled with date and time and assessed for presence of brood in all phases of development by marking the cells with a marker on the photograph.

[4] Worldwide, different bumblebee or alternative social non-*Apis* species are commercially reared for pollination purposes and, therefore, in most regions will not require import procedures (Mader et al., 2010b).

9.4.2.3 Semi-Field Studies—Stingless Species

The stingless bees (Meliponini) consist of approximately 24 genera of bees with around 400 species (the number is not clear as many species still remain to be described). They are important social bees in the subtropics and tropics (Nogueira-Neto, 1997). Meliponini occur mainly in Neotropical America, Australia, Indonesia, Malaysia, India, and Africa (Proni, 2000). These bees are and have been important cultural components of many communities in the tropics and they are managed for their pollination services and honey production.

Stingless bees have varied nesting sites, from aerial parts of trees to underground. They differ from *Apis* spp. in that their combs/cells are arranged horizontally and are mass provisioned by the nurse bees with nectar, hypopharyngeal gland secretions and pollen before the queen lays the egg after which the cell is closed. Full development ~~to~~ the adult takes place within these cells without any further input by the nurse bees; hence each cell is representative of the conditions that existed during the construction and provisioning of the cells. A newly emerged bee destroys its cell immediately. Honey and pollen stocks are usually stored at the periphery of the nest with the brood in the middle of the colony. However, the arrangement of the brood and storage pots varies between species, and for many species these details remain unknown. It is believed that the adult workers have a similar life span to that of *A. mellifera*, that is, they live 30–40 days.

Meliponini range in length from 1.8 to 13.8 mm (Michener, 2007) and, because of this, the choice of the species is important for risk assessment tests. For example, in the past few years, *Melipona scutellaris* has been tested in greenhouses on tomato plants; and in tropical areas, some species such as *Trigona carbonaria* live and/or are managed in semi-domesticated situations. See Table 3.1 for a list of species and references for non-*Apis* species that have been employed in laboratory and/or field tests.

Individual bees or the inner colony are easily accessed for testing. Individual bees can be chilled for several minutes in a freezer to slow down their movement for ease of handling (the entire hive box should not be chilled). Heard (1999) and others have developed various hive box systems that can be used to manage these bees.

As regard to size of semi-field study, it is proposed that the approach used for the honey bee be adopted for the stingless non-*Apis* species.

Key Outputs Details are similar to *Bombus* above.

9.4.3 Interpretation of Effects in Semi-Field Studies

As stated at the outset of this chapter, the interpretation of effects (i.e., a statistically and/or biologically significant difference from the control) is linked to the protection goals and, in particular, whether the results indicate that protection goals are likely to be met or not.

If the protection goal is pollination activity and/or function, then a semi-field study with measurements of foraging activity is capable of determining whether pollination activity is related to treatment. If there is an adverse effect on foraging activity in the semi-field study, then further information is required to determine whether the effects are realized at the field level. It was the view of the Workshop participants that this would be best addressed via a field study. Alternatively, consideration of risk mitigation may be elements of consideration in determining how to proceed.

If the protection goal is honey production, then the results from a semi-field study can be interpreted as follows:

- If effects are clearly *not seen* on any parameters then it can be inferred that there will be no impact on honey production at the field scale when full-sized colonies are exposed. This assumes that long-term effects from short-term exposure are not an issue.

- If effects *are seen* or observed, for example, mortality or reduction in foraging or behavioral effects, then it may not immediately be assumed that honey production will be adversely impacted at the full-field scale. Since the semi-field test is potentially a worst case exposure scenario, the assessor needs to determine whether similar or any effects would be realized at the full-field level and hence whether honey production could be impacted.

If the protection goal is maintenance of biodiversity in terms of the ecosystem service of pollination by other non-*Apis* bees, then no negative impact on populations is the protection goal. Semi-field studies showing statistically significant effects that are expected to result in high levels of mortality should be considered for more refined field studies.[5]

9.4.4 Assessment of the Variability and Uncertainty in an *Apis* Semi-field Study

As with any experimental testing, there are sources of variability and uncertainties associated with the studies. Confining organisms to a restricted study environment can confound efforts aimed at reflecting more environmentally realistic conditions. In Table 9.3, some of the sources of variability and uncertainty are discussed. To the extent that researchers can recognize and limit these potential confounding effects, the data generated from semi-field studies will likely improve, as well as their utility in regulatory decision making.

9.5 DESIGN OF A FIELD STUDY

9.5.1 When would a field study be appropriate?

Field trials may be carried out if an acceptable risk is not estimated by either lower tier tests or the proposed risk mitigation is undesirable. Questions to be answered from a field test should be based on the results of lower tier studies, whether laboratory or semi-field. For example, if behavioral effects are observed in a semi-field study, it may be desirable to see if these are observed under more realistic field conditions. It may also be more appropriate to conduct a field study where a semi-field study is not considered to be appropriate (i.e., it is not necessary always to follow the tiered approach). For example, it may be relevant when there is the likelihood of long-term effects following short-term exposure. As with any test involving animals, the need for and intent of the study should be clearly articulated. This is particularly true for field pollinator studies given the number of variables that must be managed, and the considerable resources they require both on the part of the regulated community to conduct the study as well as the regulatory authority tasked with reviewing the study.

9.6 OUTLINE OF A FIELD STUDY FOR *APIS* AND NON-*APIS* SPECIES

9.6.1 Design of a Field Study for *Apis mellifera*

Field trials can be used to address a range of exposure scenarios and effects. The results can be used by the risk assessor to determine whether significant uncertainties have been sufficiently addressed and if the protection goals may be met. However, there are various strengths and weaknesses of field studies that need to

[5] In determining whether the protection goal of maintaining biodiversity has been met, it is necessary to determine whether it is possible to extrapolate from studies on one non-*Apis* species and conclude whether the pollination services (and any other services) that are supplied by non-*Apis* bees have been adversely affected. Further work is required to develop an appropriate risk assessment scheme around those goals and hence address issues such as the potential to extrapolate from one non-*Apis* species to others.

TABLE 9.3
Variability and Uncertainty in Semi-Field Studies with *Apis Mellifera*

Parameter	Discussion of Uncertainty
Enclosed population of bees	Under natural conditions, bees are free flying; enclosing them introduces a stressor that could lead to uncertainty in interpreting the results from a semi-field study. Enclosing bees in a semi-field setting causes two main issues, which may raise uncertainty when interpreting the results—(i) effects on behavior and (ii) availability of food and therefore, on foraging activity. Food availability and foraging issues can be addressed through design considerations to ensure sufficient food is available. This can be achieved by balancing the size of the colony with the size of the enclosed crop. Details regarding possible colony size and area of crop combinations are discussed above. Providing a study designed to ensure that ample food is readily available and that there are comparable controls should account for this potential confounding variable. Enclosing the bees in a semi-field setting could translate into behavioral effects, which could reduce exposure. For example, some bees will try to forage outside and as a result remain on the tent/cage wall rather than in the treated crop. It is not known what proportion of bees will exhibit this behavior. If the compound does not exhibit repellency effects on bees, it is thought that the same proportion of bees will potentially exhibit this characteristic in the controls as in the test groups. As there will be a proportion of bees that will not be exposed then this could potentially *underestimate* the risk. However, it is also not known what proportions of bees in the field are not exposed to the pesticide, that is, the proportion that will forage elsewhere. Provided that the population size is measured as a parameter, significant differences in comparison to controls indicate whether it is treatment related or not. It is considered that on the one hand, exposure is confined and controlled; however, there will be a proportion of bees that try to forage elsewhere. Overall, participants of the Workshop believe that this parameter is likely to over-estimate potential risk, that is, it will be worst case.
Size of colony	The colony of bees that is used in semi-field studies is small compared with those used in the field; and the way that a small colony reacts is different than the way full-size colonies react. Extrapolating effects related to mortality and sub-lethal behavior from a small colony to a standard colony is uncertain and should be approached with caution. Due to this uncertainty, if any effects are noted then further studies should be considered.
Measure of mortality	Due to the confined nature of the study, it is likely that a semi-field study will yield a relatively accurate assessment of mortality. This is in contrast to the field, where detecting an accurate level of mortality within the crop is more difficult.
Density of bees in the treated crop	It is likely that the density of bees will be higher in a semi-field study compared to the field study. Due to the potential higher density of bees in a semi-field study compared to the field situation where alternative sources of food will be available, it is considered that bees are likely to have a higher level of exposure in a semi-field study, and therefore a semi-field test potentially over-estimates any effect.

TABLE 9.3
(Continued)

Parameter	Discussion of Uncertainty
Representativeness of the study site, agricultural practices, and conditions	It is unlikely that there will be a study to represent every crop and geographical and agricultural combination being considered in the specific regulatory context. Hence, there will be uncertainty regarding the representativeness of the selected study site in comparison with possible combinations under regulatory consideration. Ideally the study site, in terms of weather, flower availability, and forage, should be designed to ensure that the bees are exposed. Uncertainty regarding the representativeness of the crop will be reduced if a surrogate is chosen that ensures that bees are suitably exposed. Addressing uncertainty based on agricultural and geographical variability is more problematic.
Residues in pollen and nectar	For pollen and nectar residue sampled from the plants, there is no reason to believe that these should vary any more or less than what would occur under field conditions, with the exception of no or limited exposure to rain (wash-off), wind, or dew. Typically, semi-field studies have some latitude to make applications during periods of good weather. If poor weather is anticipated, then applications may be delayed several days provided the colonies are not already in the enclosure. However, semi-field studies are intended to reflect real world conditions, and if it rains, then such studies can still provide useful information. Typically, residue studies are conducted on the treated plants and in pollen/nectar to ensure that some level of exposure is achieved and the results are expressed relative to these residues.
Collected nectar, pollen pellets, ~~beebread,~~ and dead bees	Regarding nectar, there may be a high turnover rate (foragers) in a semi-field study and, therefore, there may be difficulties in extrapolating this information to the field situation. Pollen and associated residues should be representative of what is likely to occur in the field and, therefore, the uncertainty associated with this parameter is low. ~~Beebread~~ is difficult to collect in a semi-field study and the study has to be managed to ensure that this occurs. There is, therefore, some uncertainty regarding this parameter compared to what would happen in the field. Uncertainty exists if the study is extrapolated to other crops; for example, if one crop produces pollen and nectar whereas another species produces only pollen.
Assessment of the brood	This is possible only via OECD 75 and associated procedures.
Overall	Due to the confined nature of the semi field study, there is high confidence that exposure will occur compared to a full-field study. It is also likely that any adverse behavioral effects will be seen. Therefore, if either increased mortality compared to the control or behavioral effects are not observed then it is considered highly likely that these will not occur in the field. Uncertainty exists regarding the potential effects on brood development; however, it is considered that this will lead to potential overestimation of the risk. Due to the duration of the exposure in the semi-field study, determination of long-term effects requires special consideration.

TABLE 9.4
Strengths and Weaknesses of Field Studies for Both *Apis* and Non-*Apis* Bee Species

Strengths

Provides a realistic exposure scenario of bees foraging on a crop, provided test plot size is sufficient.

The realistic exposure scenario is likely to allow realistic behavior of the bees.

Can be designed to be consistent with good agricultural practice/grower standard practice.

Can be designed and used to assess long-term exposure and effects.

Ecologically (field level effects) and biologically (standard size colonies) more relevant than lower tier studies.

Measurement of certain protection goals can only be, or are more accurately, determined in field studies (e.g., pollination deficit or honey production) assuming that lower tier studies are insufficient to this end.

Weaknesses

Difficulty in finding appropriate sites, that is, there are practical issues in finding a site that is sufficiently isolated from other potentially attractive crops/pesticide treatments.

Because field studies are open, controlling nutritional sources may be difficult as bees may not forage exclusively within the treated field.

Expensive to establish treatment area of a size suitable for indicating "worst case" exposure. Field studies are logistically complex and are expensive since so many factors must be accounted for.

Potential difficulty related to background levels of pesticides in the foraging area.

Difficult to use toxic standard which in turn potentially raises concerns regarding sensitivity of the test system.

Potential high level of variability including weather, mortality away from the hive, replication, and interpretation of results.

be considered before they are used in risk assessments intended for use in a regulatory context. In Table 9.4, the strengths and weaknesses of the field study are listed. Qualities of the field study, with respect to either *Apis* or non-*Apis* bee species, are relatively generic and so are listed together in one table.

9.6.1.1 Study Design Considerations

For all types of application (i.e., spray, systemic solid formulation/seed treatments/soil treatments applications).

The study should use colonies with a minimum of 10 000–15 000 foraging bees. Colonies should consist of 10–12 frames and include 5–6 brood frames. If colonies are of a different size then they should be evenly distributed between treatments. According to EPPO 170, an area of 2500–10 000 m^2 (0.25–1 ha) is recommended with a larger area proposed if the crop is not particularly attractive (e.g., 0.25 ha for *Phacelia* and 1 ha for mustard and oilseed rape). EPPO 170 also recommends that there should be a minimum of four colonies per field. It may be appropriate or necessary depending upon the regulatory question being asked, to consider the use of larger field sizes as this may provide a greater degree of realism when compared to the eventual use of the product. If larger fields are used, then more colonies may be required, depending upon the attractiveness of the crop. It is important to determine, from scientific literature, the proper colony loading rates based on the crop and size of field. In determining the size of individual fields, consideration must be given to the total number of treatments (i.e., the treated crop) and replicates per treatment (i.e., colonies per treated field).

While it is potentially desirable to use a positive control in a semi-field study, it is discouraged in a full-field study. This recommendation is based on extensive discussion among the International Commission for Plant-Bee Relationships (ICPBR) and EPPO. A negative control, however, is always required.

Participants of the Workshop agree that bees generally tend to forage on sources close to the colony, but that some bees will forage further afield and these individuals could bring additional residues into the colony.

Consequently, in order to ensure adequate isolation from other sources of pollen and nectar, the site should be located at least 2–3 km from alternative cultivated agricultural sources of pollen and nectar, including pollen and nectar from orchard trees. As regards confirming exposure, the following measurements should be considered.

- Bees/m^2—at least 5 bees per m^2 on *Phacelia* spp. or 2–3 bees per m^2 on oilseed rape and mustard (EPPO ~~170~~2). These are potentially only relevant for these crops and EU conditions and should be used with caution in other regions. It should also be noted that these densities are related to the number of colonies and size of treated area.
- Pollen identification—it is recommended to have additional colonies with pollen traps fitted. Identification of pollen can be difficult and sometimes identification only is possible to family level.

If appropriate, there should be an assessment of the degree of flowering, that is, the proportion of the crop actually in flower at any one time, for example, BBCH 60 onward for oilseed rape (see ://pub.jki.bund.de/index.php/BBCH/article/viewFile/470/420 for further details). This is particularly relevant for crops such as melons. Under certain conditions, it may be possible to manage the crop to prolong flowering so that a longer exposure period could result.

For systemic compounds, it is not possible to identify a suitable positive (reference) standard. In addition, similar to considerations with systemic compounds under a semi-field design, exposure will occur over a longer time. Therefore, the honey bees should be present during the whole flowering period of the plant. Acclimation to the pesticide will occur as soon as they are introduced into the treatment area. However, a consideration of mortality due to moving the colony is still required. One potential way around this is to compare the mortality that occurs on the untreated crop to that in the treated crop.

9.6.1.2 Pre-Application

For all application types, pre-application considerations are similar to that for semi-field studies. Refer to these sections above.

9.6.1.3 Post-Treatment Assessments

All types of application (i.e., spray, systemic solid formulation/seed treatments/soil treatments applications).

Depending upon the regulatory question being asked, it may be necessary to assess behavioral effects in the field. Mortality, however, should always be determined. While this may be done via the use of dead bee traps, these may not always be appropriate, in which case sheets or tarps outside the hive should be used.

A key issue with field studies is ensuring that sufficient exposure occurs. If possible, studies should be designed to minimize alternative forage. However, it is inevitable that there will be some alternative sources present. In order to determine whether exposure has occurred, there is a need to monitor the activity of bees within the treated crop. This can be done in several ways.

- Measuring forage activity:
  - See previous discussion on measuring foraging activity, (See similar discussion under the semi-field section)
- Measuring flight activity: aided through the use of marked bees.
- Identifying pollen from outside the colony.
- Measuring residues in pollen and nectar in bees and inside the colony. (Closely related to this point is whether the exposure that has occurred will be representative of the wide-scale use of the pesticide.)

9.6.1.4 Results

The following measurement endpoints and outputs are possible from a field study.

- Colony strength: ascertained through measurements of foraging activity, flight activity, and number of dead bees.
- Weight of the hive.
- Pollen, honey, and nectar stores: ascertained through measurement of percent comb coverage.
- Mortality at the hive: ascertained through measurements with dead bee traps or collecting sheets.
- Mortality of drones and pupae: ascertained through visual inspection of frames.
- Mortality in the crop: ascertained through collection sheets in the treatment site.
- Presence of the same queen.
- Foraging activity in the crop: measured in the test crop, or at the hive entrance where it can be recorded automatically.
- Returning foraging bees: can be counted automatically at the hive entrance.
- Behavioral abnormalities.
- Measurement of residues in pollen/nectar, or via pollen pellets, as well as in wax, ~~beebread,~~ and dead bees: measurements of exposure inform assessment of risk.
- Assessment of the brood: see PO 75; this measurement may also include an estimate of the number of adults, the area containing cells, eggs, larvae, and/or the capped cells.
- Disease and/or pest levels.

9.6.2 Long-term Risk to Honey Bees from Short-term Exposure

If potential overwinter effects are identified during the problem formulation step, then it is proposed that the field study can be modified in order to examine measurement endpoints that will address this uncertainty. (Generally, field studies are more appropriate to assess the impact of overwintering than extended semi-field studies.)

If a field study is to be conducted to determine whether the use of a product has any adverse effects on overwintering survival, then it is proposed that in addition to the considerations discussed above, the following points are also considered.

Following the exposure phase, the colonies (treatment and controls) should be relocated to an area that has limited, or no agricultural crops but an abundance of natural vegetation. This is necessary to ensure that exposure to additional pesticides does not occur.

At the end of the winter period, it is proposed that the following assessment endpoints should be determined, (the exact endpoints however, will depend upon the issues highlighted in problem formulation).

- Condition of the colonies/colony strength,
- Brood development,
- Brood assessment, including:
  - Number/density/pattern of brood
  - Presence of healthy egg-laying queen
  - Estimate of pollen and nectar storage areas
  - Estimate of areas containing eggs, larvae, and capped cells
- Analysis for disease, (e.g., *Nosema apis*, *Varroa destructor*, American foulbrood, bee viruses)
- Weight of the colonies,
- Residue samples from the hive (e.g., pollen, wax, honey, bees).

9.6.3 Interpretation of Effects

As for semi-field studies, the interpretation of effects is linked to the protection goals. It should be noted that while a full-field test is the highest tier of testing it is important that final determination of potential risk and whether the use of the compound is consistent with protection goals should be based on the entire body of evidence across all tiers.

If the protection goal is pollination activity or pollination function, then the full-field study is capable of determining whether this is achieved via use of measurements on (i) foraging (which can include foraging for nectar and pollen), (ii) behavior and, (iii) mortality. If no effect is observed on any of these parameters then the protection goal will be met. If effects are seen on any of these parameters, it may be unlikely that the protection goal will be met. Risk mitigation measures may enable the protection goal to be met; it is, however, essential to ensure that an assessment of the appropriateness and practicality of the risk mitigation measures can be made, and that the protection goal is met. (It should be noted that none of the measurement endpoints directly measure pollination activity *per se*, but are surrogate measures and indicative of pollination activity. That is, in using foraging activity it is assumed that a decrease in foraging activity will result in a decrease in pollination; e.g., decreased fruit set.)

If the protection goal is honey production by the colony, then this study can provide useful information. For example, if there are clearly no effects (either biologically or statistically) then it can be inferred that there will be no impact on honey production. If significant effects are observed over the course of the study, then it may be appropriate to explore risk mitigation measures to determine whether the protection goal of honey production can be met.

9.6.4 Design of a Field Study for Non-*Apis* Bees

Given the lack of investigation into a field level test for non-*Apis* species, it is assumed that all non-*Apis* bee testing will be in conjunction with field studies that are designed primarily for *Apis* bees.

Outlined below are draft protocols that could form the basis of field studies conducted to address specific regulatory questions.

9.6.4.1 Field Studies—Solitary Bees

M. rotundata will be used as the descriptive species in this section. It is also important to note that *M. rotundata* and *Osmia* spp. have a very restricted foraging range (approximately 300 m) compared to that of *A. mellifera* (2–3 km); therefore, it is much easier to ensure that their foraging will be restricted to the crop at the study sites. Preparation of *M. rotundata* for these studies should be undertaken using the same maintenance and handling protocols described for *M. rotundata* in the semi-field study.

Key outputs include mortality (in the crop and at the hive/nest) foraging activity, and reproductive success (as a measure of colony health). Assessment of these endpoints is similar to that for *Apis* tests.

9.6.4.2 Field Studies—Social Non-*Apis* Species

Bombus spp. will be used as the descriptive species in this section. It is also important to note that *Bombus* spp. have a much more restricted foraging range (400–750 m) (Knight et al., 2005) than *A. mellifera* (2–3 km) so it is much easier to assure that their foraging will be restricted to the crop at the study sites. Preparation of *Bombus* spp. for these studies should be undertaken using the same maintenance and handling protocols described for this species group in the semi-field study.

Key Outputs Key outputs include mortality (in the crop and at the hive) foraging activity, and reproductive success (as a measure of colony health). Assessment of these endpoints is similar to that for *Apis* tests.

9.6.4.3 Field Studies—Stingless Species

Stingless bees (Meliponini) have a social life similar to the honey bees albeit in much smaller colonies. There is an increasing body of literature (Heard, 1999; Amano, 2004) showing the value of stingless bees in pollination of crops in tropical and temperate countries. The stingless bees are native to tropical and subtropical areas, and more than 400 species have been recorded from these regions. The ease of handling these species (small colony sizes, and hesitance to sting) makes them ideal candidates for pollination in greenhouse conditions. However, in terms of their use for pesticide tests, there is very little information and thus the information below should be taken as a guide with allowances for improvement. It is expected that this guidance document will create interests among the practitioners to develop and validate methods and create a forum for revisions in the future, if required.

Hives Hives for stingless bees are box-shaped (commercial units) but smaller compared to those of honey bees. They do not have frames but rather are hollow, containing the whole colony component. Opening the hive, therefore, should be done gently to avoid damaging/destroying the nest structure. (Honey and pollen are stored in pots made of beeswax. The pots are typically arranged around a central set of horizontal brood combs.) When the young worker bees emerge from their cells, they tend to remain inside the hive, performing different jobs. As workers age, they become guards or foragers. Unlike the larvae of honey bees, meliponine larvae are not fed directly. The pollen and nectar are placed in a cell, an egg is laid, and the cell is sealed until the adult bee emerges after pupation (i.e., mass provisioning). At any one time, hives can contain 300–80 000 workers, depending on the species.

Stingless bee colonies can be purchased from beekeepers that specialize in stingless bee production and management. Stingless bees that are currently commercially available in tropical countries include, but are not limited to: *Melipona beecheii; Melipona quadrifasciata; Trigona carbonaria; Tetragonula fuscobalteata; Scaptotrigona bipunctata; Tetragonisca angustula; Meliponula ferruginea; Hypotrigona gribodoi;* and, *Meliponula bocandei*. See Chapter 3 for details on which species are appropriate for specific countries.

Care should be taken to acquire strong colonies with sufficient workers, each with about 10 000 healthy foragers; however, this will depend upon the species used. Up to eight colonies per hectare may be used. Stingless bee hives can be placed at strategic positions similar to operating with honey bees (e.g., either in the middle or edge of the field); and hives should be sheltered with a wooden cover placed on top of the hive to avoid direct rainfall on the hive.

Stingless bees have a wide foraging range, foraging up to 2.1 km (Kuhn-Neto et al., 2009), but on average they restrict their activity to within 1 km of the colony. The isolation distance from other forage sources recommended for honey bees (2–3 km) can thus be used.

The number of individuals per hive and per unit area recommended for honey bees can also be applied to the stingless bees. However, noting that there have been no field tests of this kind done for stingless bees, there is a research requirement to validate the protocol.

Treatment Application, Sampling, Data Analysis, and Interpretation Same as for *Apis*

Key Outputs The endpoints for the stingless bees in the field tests are similar to the honey bees and include:

- Colony strength
- Hive weight
- Pollen, honey, and nectar stores

- Mortality at the hive (via the use of dead bee traps or collecting sheets)
- Mortality of drones and pupae
- Mortality in the crop
- Presence of the same queen
- Foraging activity in the crop
- Returning foraging bees
- Behavior
- Residues in pollen, nectar, pollen pellets, wax, ~~beebread,~~ and dead bees (i.e., measures of exposure)
- Assessment of the brood (including an estimate of adults, the area containing cells, eggs, larvae and capped cells)

9.6.5 Assessment of the Uncertainty in a Field Study

Unlike lower tier studies with insect pollinators, environmental conditions are far less easy to control in full field studies. Additionally, although sources of variability and uncertainty may exist, there may be fewer options available for researchers to address these issues under full field conditions. While many of the options available for semi-field studies may apply to full field studies, the logistics of stratifying designs and increasing the number of replicates become logistically difficult to implement. Table 9.5 highlights uncertainties associated with field level studies with both *Apis* and non-*Apis* bee species.

9.7 ROLE OF MONITORING AND INCIDENT REPORTING

Some countries have incident monitoring ~~schemes~~ aimed at providing information that can inform regulatory decisions. These ~~schemes~~ provide some feedback on the quality and accuracy of regulatory decisions and, therefore, by association elements of that decision such as measurement endpoints, assessment endpoints, up through protection goals. In addition, some regulatory authorities require monitoring of bee colonies as a condition of registration where there is uncertainty whether the risk, or the risk mitigation meets the protection goals.

Monitoring ~~schemes,~~ for example, the U K Wildlife Incident Investigation Scheme (WIIS), rely on incidents being reported to a central organization. This ~~scheme~~ has provided much information on incidents associated with correct use, accidental incorrect or misuse, as well as abuse of pesticide products. These data, along with usage data, have been useful to determine the appropriateness of various regulatory restrictions as well as to provide information on the appropriateness of the regulatory trigger values (see Aldridge and Hart, 1993, and Mineau et al., 2008). In North America (under the United States Environmental Protection Agency (~~US~~EPA) system), pesticide registrants are required to report (adverse) incidents or adverse impacts from the use of their compound~~/~~products when they become aware of them. Other stakeholders may also report incidents to the ~~US~~EPA.

These ~~schemes~~ do, however, have limitations in that they are rely on the public to both find an incident and to report it. This can potentially lead to under-reporting if the beekeepers fear retribution, or the citizen is unaware of the process of reporting. The conditions of commercial agriculture verses that of native wildlife bias reporting toward *A. mellifera*. Consequently, incidents involving non-*Apis* bee species may be under-recorded. Nonetheless, monitoring ~~schemes~~ are a useful tool to the regulator to better understand the use and effects of pesticide compounds. Cost-effective reporting ~~schemes~~ need to be developed that provide incentives to applicators to help increase reporting of experiences from the field. This is critical for improving risk assessment and mitigation.

TABLE 9.5
Variability and Uncertainty in Field Studies with *Apis* and Non-*Apis* Bee Species

Parameter	Discussion of Uncertainty
Exposure	Uncertainty of exposure should be minimized by proper location of the site in relation to other foraging sites, ensuring that the target crop is maximally attractive to bees. Determination of exposure can be made through measurements (as discussed above for *Apis* species). As with *Apis* tests, it is essential that there is information on the degree of exposure in determining the usefulness of the study.
Location of sites	The location should be relevant for the crop and environmental conditions (climatic, botanical, and edaphic) both when and where the study is conducted. The likely reality is that tests cannot be conducted for all crop/formulation/geographic combinations and so there may be uncertainty when extrapolating the results. The uncertainty could over- or under-estimate the risk depending upon the actual study in question and the uses/situations to which it is being extrapolated.
Difference between the treatment areas and the controls	It is possible that the control and the treatment areas may differ both in terms of climate and edaphic conditions. Any differences in the testing environment (i.e., vegetative surroundings, climatic, or soil conditions) should be minimized.
Extrapolation between different varieties and sub-species of bee	Only one bee species or subspecies will be tested in one study. Uncertainty will exist when extrapolating inter-species, but may also exist when extrapolating intra-species. For example, while there is information indicating that effects on *Apis mellifera mellifera* and *Apis mellifera carnica* are minimal, that is, they are of relatively similar sensitivities, the differences in sensitivity between *Apis mellifera scutellata* and subspecies of European honey bee are unknown, and *Apis mellifera scutellata* may be more or less sensitive than the European honey bee.
Mortality away from the hive	Measurement of mortality away from the hive will be difficult and, therefore, there will be much uncertainty in this parameter. It would not be reasonable to expect that any measurement endpoint can be thoroughly documented and in most cases, the best the study can do is detecting the relative differences between control and treated colonies. Dead bee traps are likely prone to the same biases in control and treated fields. It might be argued that predatory/scavenger insects may be reduced in treated fields relative to untreated fields and that there is a lower likelihood that dead bees may be removed from traps whereas in control fields greater scavenging may occur, making it appear as though mortality was lower in the untreated field. This underscores the need to calibrate dead bee traps to determine the efficiency of recovery. This parameter will potentially underestimate any level of mortality. However, other measurements, for example, colony health (strength and weight), will provide an indirect measure of mortality (i.e., if much mortality occurs away from the colony then it is likely that the overall hive health/colony development etc. will be ~~adverse~~ affected.)
Overall	A field study is an assessment of the potential effects on the colonies under more realistic climatic, botanical, and growing conditions. There are uncertainties regarding the degree to which bees are exposed, although the resulting exposure is likely to represent more normal conditions than those in a semi-field studies. There are uncertainties regarding the sensitivity of the bees tested as well as extrapolating the study to other sites, situations, and crops; however, these should be assessed on a case-by-case basis.

9.8 SUMMARY

Semi-field and field studies for *A. mellifera* are key components of the risk assessment process. They permit a further, more realistic and representative assessment of the potential risk and impacts from the use of pesticides. Due to the fact that these studies are higher tier, there are no standard guidelines available as there are for lower tier studies (e.g., the OECD acute oral toxicity 213). Each should be designed to address the concerns highlighted in the risk assessment. Currently in pesticide registration, semi-field and field studies tend to be conducted according to EPPO 170 or OECD 75. This chapter has built on the information in these guidance documents and provides further information and improvements regarding the conduct of semi-field and field studies. Information is also provided regarding how these studies can be interpreted and hence linked in a qualitative manner to protection goals. Areas of improvement are also included, with the main one being the use of statistics. While statistics are recommended in both EPPO 170 and OECD 75, no particular methodologies are proposed. This issue is further developed through other efforts connected with this Workshop.

Information is provided in this chapter on the design of semi-field and field studies for non-*Apis* bees. Due to the state of knowledge, these are not as well developed and are not currently incorporated into regulatory risk assessment. However, information in this chapter provides a useful starting point regarding what species can be tested under semi-field and field conditions and the design of such studies.

Au: Please note that we have removed the reference "Babendreier et al., 2004" from the reference list as per your instruction in Chapter 9. Also, this reference had been deleted from Chapter 7 as instructed earlier. If this reference needs to be included in Chapter 7, please provide citation in the text for this reference in Chapter 7.

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